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WNT5A Expression in Human Breast Cancer

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Abstract. *The Wnt family encodes secreted signaling molecules involved in cell adhesion and, by implication, cell growth. Wnt5a has been shown to behave as a putative oncogene and also as a tumour suppressor gene. This is a reflection of its role within a multi-step pathway and in the variety of ways in which its production can be stimulated or switched off. Wnt genes can be functionally separated into two classes; those that activate the canonical Wnt/beta-catenin pathway and those that activate the Wnt/Ca++ pathway. Wnt5a signals through frizzled receptors and, depending upon which frizzled receptor is present, may activate either pathway. Therefore the observed function of Wnt5a is entirely dependent upon its context, hence the confusion over its role in tumorigenesis. This study examines Wnt5a mRNA expression using RT-PCR in human breast cancer. Materials and Methods: One hundred and twenty malignant breast tumours and 33 normal breast tissues were analysed. The levels of transcription of Wnt5a were determined using real-time quantitative PCR. Levels of expression were analysed against staging, nodal involvement, grade, distant metastasis and survival over a 6-year follow-up period. Results: Levels of Wnt5a mRNA were lower in tumours than in normal tissue (mean values : 107 vs. 62.7). They fell further with increasing stage using the Nottingham Prognostic Index. This became statistically significant when NPI3 was compared to normal tissue (p=0.043, t-test). There was a trend towards lower levels of Wnt5a in those with progressive disease, however, this did not reach statistical significance. In patients with ER-negative disease, lower levels of Wnt5a were significantly associated with a worse clinical outcome (p=0.016). Conclusion: There is a trend for mRNA levels to be lower in cancerous tissue and lower still in those showing more aggressive behaviour. This is consistent with the hypothesis that Wnt5a is a tumour suppressor gene with potential clinical applications.*

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The Wnt family participate in a diverse range of functions during embryogenesis and tumorigenesis. They encode secreted signaling molecules involved in cell adhesion and by implication cell growth. Wnt5a has been shown to behave as a putative oncogene and also as a tumour suppressor gene. This is a reflection of its role within a multi-step pathway and in the variety of ways in which its production can be stimulated or switched off.

Wnts can be functionally separated into two classes; those that activate the canonical Wnt/beta-catenin pathway and those that activate the Wnt/Ca++ pathway. Wnt5a signals through frizzled receptors and, depending upon which frizzled receptor is present, may activate either pathway. Therefore, the observed function of Wnt5a is entirely dependent upon its context, hence the confusion over its role in tumorigenesis (1).

Wnt5a has been implicated in both oncogenesis (2-4) and suppresion (5-10) of human cancer. This study examines Wnt5a mRNA expression using RT-PCR in the context of clinical outcome in breast cancer.

Materials and Methods

Materials. The RNA extraction kit and reverse transcription kit were obtained from Abgene Ltd. (Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized in house. Master mix for quantitative PCR was from Abgene.

Breast cancer tissues (n=120) and normal background tissues (n=33) were collected immediately after surgery at two hospitals and stored at -80˚C until use. Breast cancer cell lines, stromal fibroblasts and endothelial cells were also used. Patients were routinely followed clinically after surgery. The median follow-up period for both groups was 72 months. One of the involved hospitals has accrued 120 months median follow-up; this group underwent additional analysis for survival outcome. A Consultant Pathologist (A.D-J.), who examined H&E-stained frozen sections, verified the presence of tumour cells in the collected tissues.

Details of histology were obtained from pathology reports. Follow-up data was recorded in a custom database (see Table I).

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut at a thickness of 5-10 μm and were kept for routine histology. An additional 15-20 sections were mixed and homogenised using a hand held homogeniser, in ice-cold RNA extraction solution. The concentration of RNA was determined using a UV

Table I. *Clinical data.*

Parameter	Number
Nodal status	
Node-positive	65
Node-negative	55
Tumour grade	
1	23
2	41
3	56
Tumour type	
Ductal	88
Lobular	14
Medullary	2
Tubular	2
Mucinous	4
TNM staging	
TNM 1	69
TNM 2	40
TNM 3	7
TNM 4	4
Clinical outcome	
Disease-free	87
With metastasis	6
With local recurrence	5
Died of breast cancer	16
Died of unrelated disease	6

Table II. *Levels of Wnt5a mRNA and tumour stage and clinical outcome.*

Parameter	N	Wnt5a transcripts Mean±SD	Wnt5a transcripts normalized against CK19 Mean±SD
Normal tissue	33	37.0±83.0	107.3±369.6
Tumour tissue	120	27.8±136.4	62.7±373.6
NPI1	58	32.2±174.4	48.0±336.0
NPI2	34	34.1±97.8	35.5±123.2
NPI3	15	5.72±14.34	202±766
Grade1	19	84.2±301.5	164±587
Grade2	36	20.2±83.5	5.16±15.98
Grade3	55	14.25±45.38	67.7±407.2
TNM1	62	36.1±178.5	51.4±327.8
TNM2	34	17.51±50.02	22.3±106.3
TNM3	7	26.6±67.7	0.483±1.238
TNM4	4	0.610±1.207	1.09±2.17
Disease-free	87	27.8±148.9	77.1±434
Alive with metastasis	6	18.0±30.3	14.0±33.8
Local recurrence	5	0.0±0.0	0.0±0.0
Died of Br Ca	16	46.8±135.0	43.3±153.3

spectrophotometer. Reverse transcription was carried out using a reverse transcription kit with an anchored olig (dT) primer supplied by Abgene, using 1 μg of total RNA in a 96-well plate. The quality of cDNA was verified using β-actin primers.

Quantitative analysis of WNT5A. The level of WNT5A transcripts from the above prepared DNA was determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported previously (11).

PCR primers were designed using Beacon Designer software but to the reverse primer an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK), was added.

The reaction was carried out using the following: Hot0start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol of FAM tagged probe (Intergen Inc.) and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (BioRad) which is equipped with an optic unit that allows real-time detection of 96 reactions under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 sec, 55˚C for 40 sec and 72˚C for 20 sec. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. Levels of Wnt5a expression were then normalized against CK19 expression already measured in these specimens, to correct for varying amounts of epithelial tissue between samples. The primer sequences were as follows: the forward primers: wnt5aF1- catgaacctgcacaacaac and the reverse primers: wnt5aZr – agcatgtcttcaggctacat

The product size was 108 kb. Primers used for quantitation of ER and ER-β was as we previously reported (13): ER, 5'cctactacctggag aacgag'3 and 5'ctcttcggtcttttcgtatg'3; and ER-β, 5'aaaagaatcattcaa tgaca'3 and 5'attaacacctccatccaaca'3. Primers used to quantify CK19 was as previously reported (14): 5'-caggtccgaggttactgac-3' and 5'-actgaacctgaccgtacacactttctgc cagtgtgtcttc-3', respectively (12,13).

Results

The raw data showed that levels of Wnt5a were higher in normal tissue than cancerous tissue. Breast cancer cell lines and stromal fibroblasts expressed Wnt5a. The non-metastatic MCF-7 expressed higher levels of Wnt5a that the aggressive MDA MB 231. Most tumour samples contained only low levels of Wnt5a and, therefore, the mean values were used for statistical analysis rather than the median as these were almost all 0. The Wnt5a levels were also normalised against CK19 (an epithelial marker) to correct for the varying amounts of epithelial tissue in each sample (Table II).

Those patients with positive nodal status (n=49) showed lower levels of Wnt5a than normal tissues, however, this was not statistically significant.

The tumour stage (NPI and TNM) was determined in 107 patients and the tumor grade was known in 110 patients. It can be seen from the raw data that levels of Wnt5a fall with increasing grade of tumour, but this was not statistically significant. Levels of Wnt5a fall with increasing stage using the TNM classification, although this does not become statistically significant, $p=0.11$.

There was a trend towards lower levels of Wnt5a expression with increasing score using the Nottingham Prognostic Index, which becomes significant when NPI3 is compared to normal tissue ($p=0.043$).

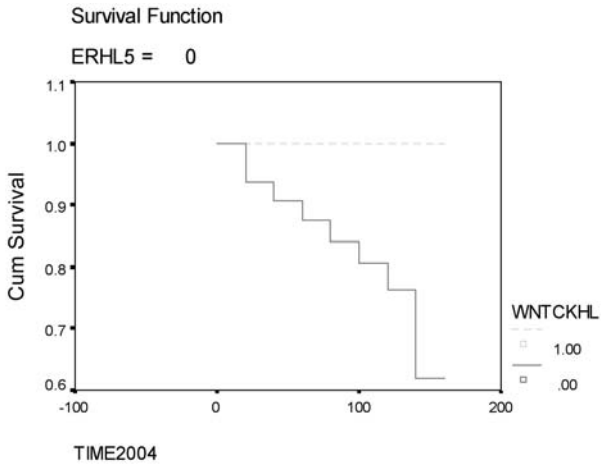

Figure 1. *Wnt5a expression and survival in patients with ER-negative disease (p=0.0166)*

Clinical outcome. In a group of patients from one study hospital, the median follow-up has reached 120 months (mean 105 months). Therefore Wnt5a expression and survival in these cancers was analysed separately. For this analysis an arbitrary cut-off value was assigned to Wnt5a expression and expression was scored high or low. The Kaplan-Meier curve showed a significant relationship between Wnt5a expression and survival in patients with ER-negative tumors after CK19 normalisation (p=0.0166) (Figure 1). The green line represents those who scored high and survived and the red line represents those with low expression and poor outcome. There was a similar trend in ER-positive tumours, but this was not significant.

Table III shows the relationship between Wnt5a levels and clinical outcome. When ER-negative and -positive tumours are analysed together; there is no significant relationship between survival and Wnt5a expression (see Table IV).

Discussion

Our results demonstrate a trend for mRNA levels to be lower in cancerous tissue and lower still in those showing more aggressive behaviour. This is consistent with the hypothesis that Wnt5a acts as a tumour suppressor gene in breast cancer. These findings are consistent with several previously published studies (5-10).

Liang *et al.* (5) showed that Wnt5a signaling through the non-canonical Wnt/Ca2++ pathway suppresses cyclin D1 expression and negatively regulates B cell proliferation. They showed that Wnt5a hemizygous mice developed clonal myeloid leukemia and B cell lymphomas with loss of Wnt5a function in tumour tissues and, further to this, that human primary leukemias also showed loss of Wnt5a function and/or deletion of the WNT5A gene demonstrating that Wnt5a suppresses haemopoietic malignancy (6, 7).

Table III. *Levels of Wnt5a were less in those patients who had recurrent disease or died.*

		Wnt5a QPCR values		Wnt5a QPCR normalised against CK19	
Parameter	No.	Mean±SD	*P* value mean *vs.* normal tissue (*t*-test)	Mean±SD	*P* value mean *vs.* normal tissue (*t*-test)
Normal tissue	33	37.0±83.0		107.3±369.6	
Tumour tissue	120	27.8±136.4	p=0.63	62.7±373.6	p=0.55
Disease-free	87	27.8±148.9	p=0.68	77.1±434	p=0.71
Alive with metastasis	6	18.0±30.3	p=0.33	14.0±33.8	p=0.17
Local recurrence	5	0.0±0.0	Values too low for analysis	0.0±0.0	Values too low for analysis
Died of Br Ca	16	46.8±135.0	p=0.79	43.3±153.3	p=0.4

Table IV. *Wnt5a levels, survival and progressive disease.*

Parameter	No.	*Vs.* disease-free	*Vs.* disease-free after CK19 normalisation
Disease-free	87		
Alive with metastasis	6	p=0.64	p=0.21
Local recurrence	5	Values too low for analysis	Values too low for analysis
Died of Br Ca	16	p=0.62	p=0.59
All progressive disease	27	p=0.85	p=0.38

Jonnson *et al.,* in two papers, examined the role of Wnt5a in breast tissues. They showed in breast cell lines that elevation of Wnt5a was linearly correlated with cell density (supporting Wnt5a's role in cell-cell interactions), but that this relationship was lost in cancer cell lines. They showed that agents known to affect Wnt5a expression could up-regulate its expression in normal cells and alter cell morphology, but that this effect was lost in cancerous cell lines, suggesting that Wnt5a has a regulatory affect on growth which is lost in cancer.

In vitro experiments by other researchers support this work in that WNT5A has been shown to inhibit the effects that are

promoted by cell transformation and direct inhibition of WNT5a can cause mouse mammary cells to transform into elongated highly refractile cells that continue to replicate in dense culture. Loss of Wnt5a expression has been shown to correlate with cell transformation when C57 mouse mammary cells are exposed to ectopic Wnt1 or neu/erbB-2 oncogene. Finally ectopic expression of human Wnt5a reversed the phenotype of a mouse epithelial cell line from tumorigenic to non-tumorigenic (8, 9).

In our study, we investigated the mRNA rather than protein expression of Wnt5a. However, our findings are consistent with other studies which measured protein expression. The clinical relevance to human breast cancer has been previously explored by Jonsson *et al.,* who measured Wnt5a expression using immunohistochemical techniques and correlated expression with histology, tumour grade and clinical outcome, with a mean 14-year follow-up in those patients who remained disease-free. They showed that, in the ductal carcinomas only, loss of Wnt5a expression was significantly associated with higher histological grade, hormone receptor negativity and disease recurrence (7). Interestingly, Saitoh *et al.* (10) have reported that up-regulation of Wnt5a was seen in gastric cancer, but was almost undetectable in gastric cancer cell lines. These results may represent up-regulation of Wnt5a by the stroma in some malignancies. However, other investigators reported that Wnt5a immunostaining was definitely seen in the epthelial component of the tumour tissues studied (7). Our finding of a significant survival benefit in patients with high Wnt5a mRNA and negative ER tumours has not been previously reported and requires further research.

The evidence for Wnt5a in oncogenesis and tumour progression comes from a limited number of studies (2-4). Two American studies on Wnt5a in melanoma have shown varying results. Reduced levels of Wnt5a were associated with more aggressive histological features in a study by Pham *et al.* (2). They showed that Wnt5a was expressed by normal cells and melanomas with small uniform cells, but that melanomas with large pleomorphic cells showed low levels of Wnt5a. Weeraratna *et al.* (3) showed that increased Wnt5a expression was directly correlated with increasing tumour grade, and that antibodies to Frizzled-5, the receptor for Wnt5a, inhibited cellular invasion.

In colon cancer, Wnt5a may be involved in the progression from normal to cancerous mucosa, since there was slightly increased expression seen in colon cancer although it was also present in normal mucosa particularly at the bases of crypts. They also noted that the Fz receptors 1 and 2 were not seen in normal tissues or well-differentiated tumours, but were over-expressed in poorly-differentiated tumours, particularly at the margins of invasion (4).

It is likely that Wnt5a has several functions of which tumour suppression is one. It may also act synergistically with other known tumour suppressor genes (TSGs) on chromosome 3, whose action may be more prominent (14). This potential tumour suppressor role of Wnt5a in breast cancer has clinical implications in terms of clinical outcome prediction and therapy and requires further evaluation in validation studies.

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